



Case report

Determination of cocaine and its major metabolite benzoylecgonine in several matrices obtained from deceased individuals with presumed drug consumption prior to death



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ABSTRACT

In the field of forensic toxicology, femoral blood is the most useful sample for the determination and quantification of drugs; however, cases in which blood is unavailable are common. In such cases, validated methodologies for drug determination in alternative matrices can be decisive in the investigation of a case. In particular, when femoral blood is unavailable for analysis for the presence of systemic exposure to cocaine and its principal metabolite, benzoylecgonine, validated methodologies from matrices other than blood that can be obtained in the autopsy room would be useful to the forensic toxicologist in the evaluation of a specific forensic case. To address this issue, we implemented and compared in our study the systematic evaluation of extraction, chromatographic separation, and quantification of cocaine and benzoylecgonine in different biological matrices (right and left cardiac blood, femoral arterial and venous blood, urine, vitreous humor, cerebrospinal fluid, brain accumbens nucleus, brain ventral tegmental area, and liver). The studied matrices were those most likely to be obtained from different autopsy rooms at the time of forensic testing in deceased individuals who are presumed of antemortem drug consumption. Solid phase extraction of analytes from the different matrices was performed using C-8/SCX mixed-phase columns, and gas chromatographic mass spectrometry separation was performed using detection in single-ion monitoring mode. The methodological validation was performed for all the studied matrices, and the results showed similar sensitivity and recoveries without statistical differences between the studied matrices. The methods were applied to evaluate a thanatological case using all the study matrices, showing unequal postmortem distribution of cocaine and benzoylecgonine throughout the different matrices tested. The present work opens the option of applying appropriate methodologies in the analysis of matrices, other than the usual blood, to obtain reliable results that may help clarify a forensic case. In addition, we present findings from different studies. This work affirms not only the potentiality of obtaining reliable data but also reaffirms the challenge of applying these data and taking into account the complexity of interpreting results in matrices other than blood.

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1. Introduction

Cocaine is one of the most powerful known natural stimulants of the central nervous system and is also highly addictive. The prevalence of drug consumption in Chile has decreased from 2008 to 2010, according to the Ninth National Drug Study in the General Population report in 2011 by the National Service for Prevention

and Rehabilitation of Drug and Alcohol Consumption.¹ This study showed that compared with more expensive drugs, marijuana is the most commonly consumed drug in Chile. However, the geography and boundary conditions of Northern Chile, together with the availability and lower price of cocaine in this country, have recently led to the potential consuming population among the residents in the northern zone to rapidly becoming an important percentage. This has been reflected in toxicological results obtained from multiple autopsies performed by regional forensic services.

In 2008, Iquique Forensic Service, located in a northern zone of the country and bordering the countries of Peru and Bolivia,

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performed 200 autopsies corresponding to forensic cases, from which 131 were derived to the toxicological laboratory. Of these cases, 27 (20.6%) tested positive for cocaine in the blood, according to the toxicological examination performed. Nevertheless, according to the characteristics of the forensic case or the circumstances that led to the death of the person (degree of destruction of the body of the deceased), in some occasions, it was not possible to rely on a femoral blood sample, which is the internationally recommended matrix for the testing of cocaine and its products.² Another factor that may affect postmortem studies investigating cocaine is the amount of time that has elapsed between death and sampling. This is of particular importance, as postmortem redistribution and degradation make it impossible to determine the blood concentration of cocaine to help explain the cause of death, even with certain presumption of prior use.

Given the importance of identifying the presence of cocaine and its major metabolite, benzoylecgonine (BZE), in cases of the forensic deceased, validation parameters are necessary for the accurate determination and quantification of cocaine and BZE in different matrices that could be sent from the autopsy room to the toxicological laboratory. Previous literature has described methodologies for the quantification and identification of cocaine in some matrices other than blood.^{3–13} These methods could be used in the event that femoral blood is unavailable. However, most publications investigate the matrices individually and do not identify a wider range of alternate options. Nevertheless, some publications suggest methodologies for identifying cocaine and its metabolites in several liquid matrices such as blood, plasma, urine, and oral fluid, which we used as the initial support for the development of this work.^{8,10–17} These methods have broadened the range of possibilities available to both forensic toxicologists and coroners, especially when faced with a complicated autopsy in the context of bodily integrity, in terms of the analysis of the samples that potentially available in a routine autopsy procedure.^{12–24}

In forensic toxicology, it has been proposed that cadaver blood samples be taken directly from the femoral vein after being linked to the inguinal zone to avoid contamination from the abdomen. Due to its peripheral condition, this vein is relatively less affected by postmortem redistribution.² The use of vitreous humor (VH) has also been recommended because of its scarce irrigation and the protection that the bony skull structure provides to the ocular globe, which preserves the concentration of xenobiotics, and is very close to the systemic blood circulation.^{2,25} Commonly abused drugs, particularly cocaine, have been detected in VH.²⁶ Furthermore, in an autopsy, a greater diversity of specimens can be collected, including hair, muscle, brain, fat, and lung.^{3,8,12,13,27} It is important to note that the matrix chosen for analysis is frequently dictated by the case under study and/or the condition of the deceased body. Nevertheless, the most common specimens for cocaine analyses in postmortem cases are blood, liver, and urine.^{5,8,10,11,14,15,17}

In cases of extreme putrefaction, muscle tissue, hair, and bone may be used as appropriate specimens; nevertheless, the physical state of the deceased body will determine which specimens are possible to collect.²⁷ Liver has always been a key tissue in postmortem toxicological analyses, and the result obtained from this tissue is frequently complementary to any other result obtained from blood. It must be taken into account that the diffusion of cocaine is possible from the large intestine; therefore, using deep tissue from inside the right lobe of the liver is preferred.²⁸ Brain tissue has also been used for years by toxicologists, mainly in determining the concentration of a substance in a tissue where many toxic substances exert their effects. However, given the unequal distribution of the drugs and the frequent location of action sites in the brain for the different abused drugs, accurate

interpretation is more difficult than in peripheral tissues.²⁹ However, the National Institute on Drug Abuse indicates that at the brain level, one of the neuronal systems that appear to be more affected by cocaine, the ventral tegmental area (VTA), originates in a very deep region of the brain.³⁰ Nerve cells that originate in the VTA extend into a region of the brain known as accumbens nucleus (AN). Furthermore, this report suggests that during a gratifying event, VTA neurons considerably increase the amount of dopamine released into the AN. During the normal communication process, a neuron releases dopamine inside the synaptic space. Dopamine is linked to its specific receptors in the adjacent neuron, sending a message to it. Cocaine interferes with this normal communication process mediated by neurotransmitters.^{30,35}

Currently, many published works use similar analytical procedures for cocaine assay. In operations prior to analysis, solid-phase extraction (SPE) is a widely accepted technique, which is applied to the extraction of different abuse drugs, including cocaine and its metabolites, from different matrices.^{8,11} Solid-phase micro-extraction (SPME) is also used^{15,31} with good results, but on a smaller scale than SPE owing to the higher initial cost of the inputs required and because it is not manually applicable to a great number of samples simultaneously.

The main aim of this research was the systematic comparison of cocaine and BZE determination in different matrices, considering the analytical advantages and limitations from the point of view of their application in the study of postmortem distribution of these drugs.

2. Case report

This work is mainly focused on validating methodologies for both the determination and quantification of cocaine and the principal metabolite BZE in matrices other than femoral blood. We also investigated a case where it was possible to include all assessed matrices. In this sense and as an example, the forensic case Prot 141 was considered. This case investigated a 22-year-old man who was 1.72 m in height, with a body mass index of 23.66. The autopsy and sampling data were performed 11.5 h after death. The precise circumstance of the death was asphyxiation by hanging (suicide), without apparent participation of third parties.

During the autopsy, the pathologist collected femoral venous blood, according to routine, and all of the other matrices were collected as part of this study. The final report sent from the forensic laboratory showed only the femoral venous blood results. Nevertheless, the differences in concentration in other matrices obtained from the same body became evident, as will be shown in the following section.

3. Materials and methods

3.1. Samples obtained from the human body

Samples used in this work for determination and quantification of both cocaine and BZE were right cardiac blood (RCB or venous), left cardiac blood (LCB or arterial), femoral arterial blood (FAB), femoral venous blood (FVB), urine, VH, cerebrospinal fluid (CSF), brain accumbens nucleus (AN), brain ventral tegmental area (VTA), and liver. This spectrum of samples included both solid and liquid matrices, providing multiple possibilities to the coroner at time of sampling, as well as to the toxicologist at the time of analysis.

The diverse target matrices used to implement analytical methodologies in this study were obtained in the autopsy room from donors with no history of cocaine consumption. These records were collected in the usual interviews made to relatives of the deceased, as conducted at the Chilean Forensic Service. A screening

urine analysis was performed for the deceased, in the autopsy room and before the thanatological procedure, using an immunochromatographic screening Abuse Drug Panel (10) (Inmunodiagnóstico Ltda., Santiago, Chile), which is sensitive to 10 drugs and their metabolites (amphetamine, cocaine, tetrahydrocannabinol, benzodiazepines, tricyclic antidepressants, barbiturates, methamphetamine, morphine 300, methadone, and ecstasy).³² For this panel, the limit of detection for cocaine and BZE in U is 300 ng/mL. Other metabolites, besides BZE, were not included in the study because a deuterated internal standard of ecgonine methyl ester or other cocaine metabolite was not available in Chile for method validation. Furthermore, the screening kit used to detect cocaine is sensitive to cocaine and must be calibrated with BZE; no possible cross-reactivity with ecgonine methyl ester or other drug metabolites was reported.

Methodologies were applied in cases from different autopsy rooms, with positive results in the screening test for cocaine, in urine or VH, independent of sex and age. All of the samples were obtained from autopsies performed between 2009 and 2011. All the samples, including the liquid matrices to which sodium fluoride was added, were stored and frozen at -30°C to avoid decomposition and the possible loss of analytes before processing.

3.2. Methods of cocaine and BZE extraction from different matrices

Extraction of the different analytes determined in the toxicological examination performed at the toxicological laboratories of the Chilean Forensic Service and also for the special case of this work was performed by SPE using C-8/SCX mixed-phase columns (Bond Elut Varian-Certify 300 mg), provided by Merck S.A., Santiago, Chile.

3.2.1. Pretreatment of liquid matrices

Samples (2 mL) were combined with 8 mL of phosphate buffer (0.1 M, pH 6) and 400 ng of their respective deuterated internal standards. The mixture was sonicated for 30 min and then centrifuged for 10 min at 3500 rpm; the supernatant was then passed through a previously conditioned SPE with 2 mL of methanol and 2 mL of phosphate buffer (0.1 M, pH 6). Cleanup steps using hexane-ethyl acetate 80:20 and 50:50 acetone-chloroform (2 mL of each) were performed, and the elution was performed using organic solvents of different polarity, namely dichloromethane-isopropanol-ammonia 78:20:2 and ethyl acetate-ammonia 98:2 (2 mL of each). Extracts were evaporated to dryness under nitrogen stream and then derivatized with *N,O*-bis(trimethylsilyl)-tri-fluoroacetamide with 1% trimethylchlorosilane acetonitrile 50:50 (100 μL) at 70°C for 25 min and finally injected into the gas chromatograph.

3.2.2. Pretreatment of solid or semisolid matrices (brain or liver)

The extractive methodology was performed according to the procedures proposed by Mora et al.³ This consisted of milling the sample, weighting (approximately 2 g), and later, the addition of 8-mL phosphate buffer (1.0 M, pH 6) and their respective deuterated internal standards (400 ng each). The mixture was sonicated for 1 h, then filtered through a cotton ball in a small funnel, and passed through a previously conditioned SPE, following the same procedure described previously.

3.3. Gas chromatography coupled to a mass detector

A 6890N gas chromatograph from Agilent Technologies, equipped with an 7693 autosampler, an 5975 mass spectrometer, and MS ChemStation software were used for qualitative and quantitative analyses. The capillary column used was a DB-5MS (J & W

Scientific), which was 60 m in length and 0.32 mm in diameter, with a 0.25 μm inner film, using helium 6.0 as carrier gas. The temperature program was 50°C for 4 min, $35^{\circ}\text{C}/\text{min}$ until reaching 180°C (1 min), $25^{\circ}\text{C}/\text{min}$ up to 250°C (1.24 min), and finally, $20^{\circ}\text{C}/\text{min}$ until 315°C (4 min). Next, 5 μL of sample was introduced through a PTV injector,³³ allowing a large sample volume with solvent elimination. The injector temperature program was 100°C for 1.6 min and then $700^{\circ}\text{C}/\text{min}$, until reaching 330°C in solvent vent mode, with a venting time of 1.5 min, venting flux of 150 mL/min, pressure of 30 psi, purge at 6 min, purge flux of 100 mL/min, gas saver at 10 min, and flux of 150 L/min. For the quantitative analysis of both cocaine and BZE, the SIM methodology was used, with m/z 82/182/185/303/306, and m/z 182 and 185 as quantifying ions. Ions corresponding to BZE were m/z 82/240/243/361/364, and m/z 240 and m/z 243 were the quantifying ions.

3.4. Calibration curves and validation parameters

The calibration curves were prepared for each sample of both analytes, between 10 and 200 ng/mL (liquid matrices) and between 10 and 200 ng/g (solid matrices), using 400 ng of deuterated internal standard (40 μL of a solution of 10 $\mu\text{g}/\text{mL}$). Linearity was evaluated using spiked matrices at different concentration levels. The data obtained at lower concentration levels was used for LD and LQ estimation (considering sy/x as sB).³⁴ The recovery studies were performed using the spiked matrices at different concentrations and subjected, in triplicate, to the full methodology, including sample pretreatment and GC–MS analyses; quantification was performed using the calibration curves of standards. Samples which exceeded the linear concentration range were diluted and reanalyzed.

4. Results and discussion

4.1. Comparison of analytical methods

The first step in the comparative study of the application of the extraction procedures to different solid or liquid matrices was the evaluation of their complexity. The extraction process for solid matrices (liver – AN – TVA) is not different from the extraction process for liquid matrices, except in a previous treatment where milling and filtration through cotton were used. Filtration through cotton was useful because of its ease of implementation, and unlike normal filtration paper, the method is readily reproducible and relatively quicker. The higher lipid content in solid matrices does not represent more complex extraction or cleanup steps, making them viable sample alternatives for thanatological applications. Figs. 1 and 2 present the chromatograms of positive samples for cocaine and BZE in some of the studied matrices. As can be seen in the total ion chromatograms (TIC mode), VH was the least complex matrix and VTA was the most complex; however, the selective ion monitoring mode (SIM) allows for the elimination of matrix peak interferences.

Validation parameters obtained by application of the proposed method to different studied matrices are summarized in Table 1. The calibration curves show similar sensitivity (slope) for cocaine and BZE, being this parameter independent of the sample matrices (between 0.0043 and 0.0045 for cocaine and between 0.0038 and 0.0043 for BZE). No differences between slopes of pure standard calibration curves and those prepared on matrices were observed, demonstrating the efficiency of the sample treatment together with the high selectivity of the SIM detection mode of MS. However, as expected, the complexity of matrices affected the detection and quantification limits of the method. Matrices of less complex constitution, such as VH and CSF, showed lower detection and

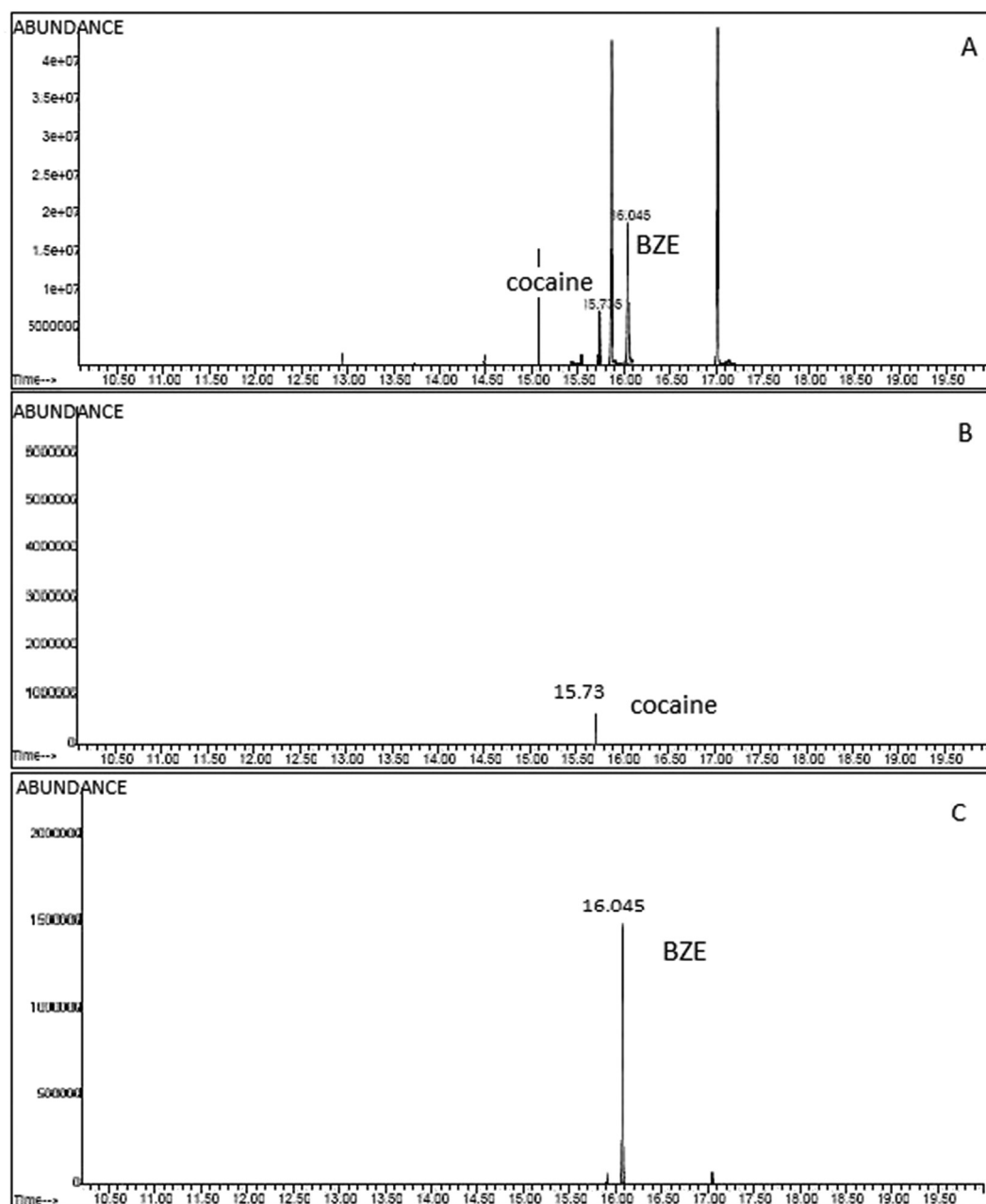


Fig. 1. Accumbens nucleus (AN). A: Total ion current, B: Cocaine: SIM at 182 m/z, C: BZE: SIM at 240 m/z.

quantification limits than did those obtained from the remaining matrices studied.

The recovery studies were performed using spiked matrices at the above-mentioned different concentration levels and subjected to the full method in triplicates, including the sample treatment. The GC–MS analysis showed recovery values higher than 92% for cocaine and BZE in all the studied matrices (Table 1). A more detailed evaluation of this process is presented in Table 2 for AN, where recoveries for cocaine and BZE were similar. The statistical variance analysis of recoveries for all the studied matrices (Table 1) showed no significant difference attributed to the sample matrices. Meanwhile, the statistical analysis results allowed the confirmation of the accuracy of determination of both cocaine and BZE in all the studied matrices, albeit with a higher variance in the determination of BZE ($p > 0.05$). This confirms the accuracy in the determination of cocaine and BZE in different biological matrices potentially

obtainable from an autopsy room during a determined thanatological process. Therefore, this process constitutes an efficient tool in the thanatological application, as it presents viable alternatives for when the forensic case does not allow another possibility (femoral blood), or even when another variable is required for the analysis of a specific case.

4.2. Thanatological application

The methodologies implemented were applied to samples obtained during autopsies from different real cases that gave positive results in the screening performed prior to thanatological process. Figs. 1 and 2 show the most representative chromatograms of one case selected from the positive cases, obtained in the autopsy room of the Forensic Service, in which all matrices submitted to study were sampled. As previously discussed, the chromatograms

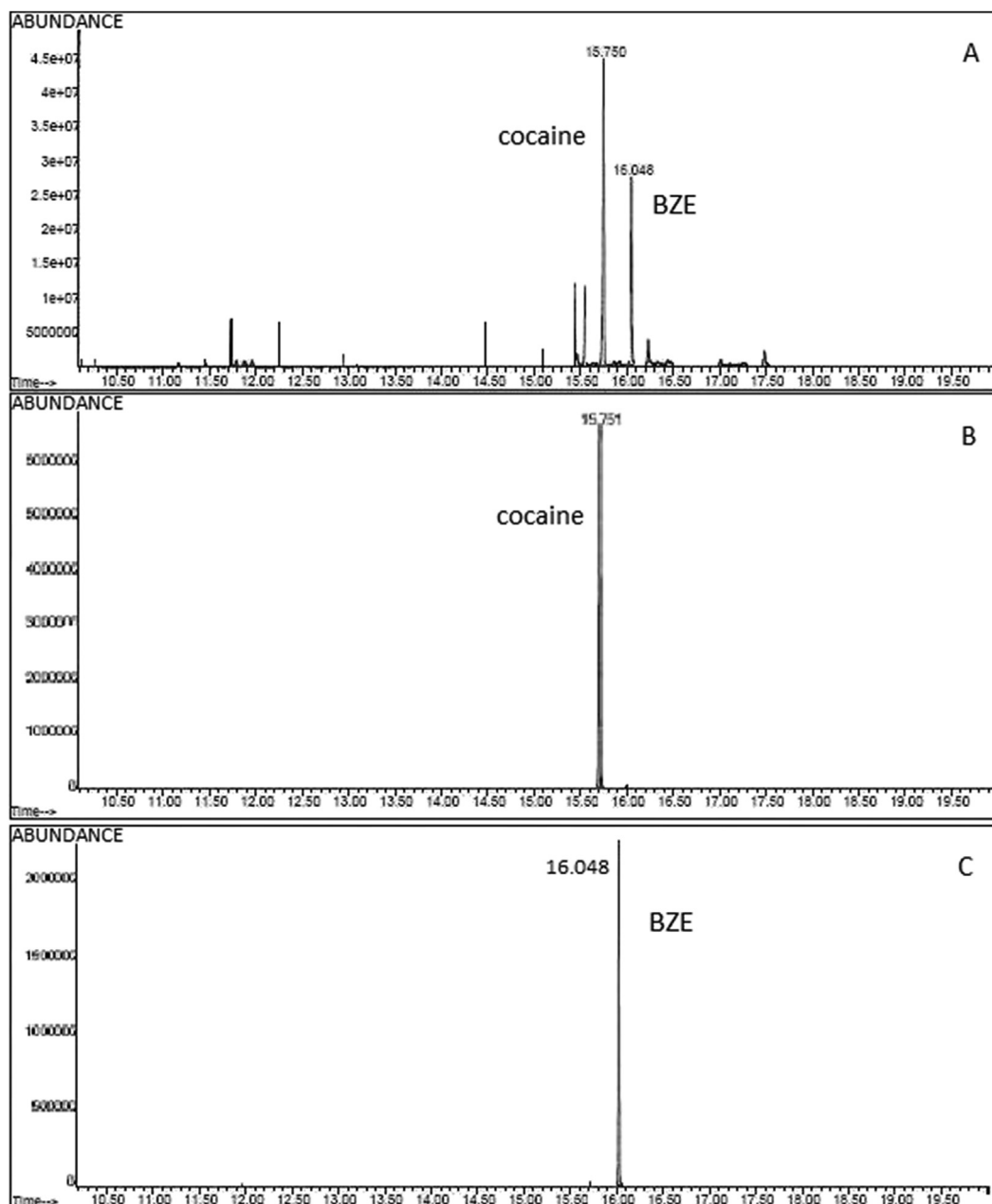


Fig. 2. Ventral tegmental area (VTA). A: Total ion current, B: Cocaine: SIM at 182 m/z , C: BZE: SIM at 240 m/z .

show sufficient selectivity for the studied matrices, displaying the total resolution between the searched analytes and the interferences of each matrix when total ion monitoring mode is used, which were improved when SIM was applied for quantitative analyses. Data obtained from such chromatograms for the selected case are summarized in Table 3, where concentrations obtained for cocaine and its major metabolite BZE in the different matrices are shown. Much higher concentrations of the sum of both drugs were found in U and in FVB than in the other studied matrices, where similar levels were observed (between 3669 and 5516 ng/mL), with the exception of liver, where lower concentrations was 976 ng/mL. In contrast, a higher BZE concentration was found in almost all the matrices, with the exception of urine and AN, where the clear predominance of cocaine (higher than 77% and 59%, respectively) was observed. These results show an unequal postmortem distribution of the original drug (cocaine)

and its major metabolite (BZE) in the different compartments of the deceased body.

Although some of the matrices studied, such as FAB and FVB, can be considered similar, the sampling applied in this study, along with the dissimilar distribution of the drug in the body of a deceased person, opens the opportunity for a deeper future study with respect to the possible distribution that cocaine and its major metabolite BZE might have in the postmortem stage.

Along with the unequal distribution found for the drug and its major metabolite, this work allowed the development and implementation of a validated analytical methodology for a series of biological matrices most likely to be obtained when a body arrives in the autopsy room. This wider range of matrices offers more options to the coroner at the time of deciding which sample will be extracted in a specific forensic case. This would make it unnecessary to solely rely on the international recommendation of

Table 1

Validation parameters for cocaine and BZE in the different matrices studied.

Matrices	Analyte	<i>m</i>	<i>b</i>	<i>R</i> ²	Concentrations range (ng/mL or ng/g)	Variation coefficient	LOD (ng/mL or ng/g)	LOQ (ng/mL or ng/g)	Recovery %
Pure standard	Cocaine	0.0044	0.0151	0.9916	(10–200)	—	0.05	0.16	—
	BZE	0.0040	0.0243	0.9985	(10–200)	—	0.73	2.42	—
Vitreous humor	Cocaine	0.0045	0.0163	0.9990	(10–200)	5.84	0.23	0.76	98.84 ± 4.04
	BZE	0.0040	0.0269	0.9939	(10–200)	10.57	0.76	2.55	96.05 ± 2.85
Blood	Cocaine	0.0043	0.0254	0.9936	(10–200)	12.40	1.51	5.03	97.88 ± 3.50
	BZE	0.0040	0.0334	0.9960	(10–200)	12.97	4.29	14.29	97.72 ± 5.18
Urine	Cocaine	0.0045	0.0222	0.9970	(10–200)	7.11	5.09	16.95	100.99 ± 3.55
	BZE	0.0042	0.0270	0.9947	(10–200)	9.97	6.00	20.00	98.92 ± 3.95
Accumbens Nucleus	Cocaine	0.0042	0.0277	0.9895	(10–200)	6.22	0.83	2.78	97.11 ± 3.67
	BZE	0.0043	0.0191	0.9899	(10–200)	10.52	4.29	14.29	97.62 ± 6.80
Ventral tegmental area	Cocaine	0.0045	0.0041	0.9995	(10–200)	5.39	1.03	3.44	92.73 ± 6.73
	BZE	0.0038	0.0333	0.9921	(10–200)	5.93	3.70	12.35	96.13 ± 1.98
Liver	Cocaine	0.0044	0.0247	0.9943	(10–200)	7.82	0.52	1.74	98.90 ± 3.70
	BZE	0.0039	0.0302	0.9912	(10–200)	11.54	1.23	4.10	96.62 ± 2.37
Cerebrospinal fluid	Cocaine	0.0044	0.0256	0.9850	(10–200)	1.86	0.19	0.62	97.94 ± 4.63
	BZE	0.0039	0.0285	0.9922	(10–200)	1.68	0.76	2.55	93.76 ± 3.46

m = slope, *b* = intercept.**Table 2**

Statistic evaluation of cocaine and BZE recovery from accumbens nucleus (AN).

Calibration curves	Concentration ng/g	% Recovery cocaine	% Recovery BZE
C1	10	103.08	98.48
	20	92.94	86.82
	50	94.50	98.66
	100	98.97	97.47
	200	96.30	106.64
C2	10	104.11	100.26
	20	94.18	87.52
	50	95.17	98.98
	100	99.15	97.77
	200	96.45	106.76
C3	10	100.45	97.40
	20	92.11	85.64
	50	94.24	98.11
	100	98.76	97.27
	200	96.22	106.53
Mean ^a		97.11	97.62
Standard deviation ^a		3.56	6.64
Variation coefficient % ^a		3.67	6.80

^a Values obtained with three calibration curves for cocaine and BZE in brain accumbens nucleus matrix.**Table 3**

Concentration of cocaine and BZE in the different solid and liquid matrices obtained from autopsy protocol 141.

Liquid samples		
Protocol 141	Concentration cocaine (ng/mL)	Concentration BZE (ng/mL)
FVB	3,210.60	19,847.00
FAB	970.4	3,031.70
RCB	1,111.50	3,458.90
LCB	1,635.90	3,116.40
VH	230.8	2,633.70
U	200,174.90	57,453.00
CSF	537.4	3,132.50
Solid samples		
Protocol 141	Concentration cocaine (ng/g)	Concentration BZE (ng/g)
VTA	2,673.10	2,842.90
AN	2,384.60	1,652.30
LIVER	1.9	973.90

femoral blood as the sample used in the toxicological forensic environment, which is often absent as a result of the physical state of the body (decomposition, deterioration, or degree of destruction). The present work provides the option for selecting the appropriate methodology to deal with the analysis of matrices other than those of the usual blood samples. Therefore, the proposed methodologies make it possible to obtain reliable results that would help clarify the forensic case under study.

Ethical approval

None declared.

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Conflict of interest

None of the authors have any conflicts of interest with respect to this study.

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